Journal of Biochemistry and Molecular Biology, Vol. 33, No. 3, May 2000, pp. 213-220

JOURNAL OF
Biochemistry
and
Molecular Biology

Inhibition of Fat-Storing Cell Proliferation by a Monomeric Arginase Derived from Perfused Rat Liver

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Reveived 19 January 2000, Accepted 9 March 2000

A fulminant hepatitis is associated with massive liver cell necrosis and a high mortality rate. But survivors regenerate a normal liver and do not have chronic liver disease. This clinical course suggests that the acutely injured livers release a factor that allows a recovery from chronic hepatitis or cirrhosis. The objective of this study was to isolate and characterize an anti-fibrotic factor from acutely damaged rat livers. The liver cell necrosis was prepared from rat by warm ischemical perfusion and the perfusates were assessed against the growth inhibition of fat-storing cells (FSC). A liver-derived growth inhibitory factor (LDGIF) was purified from ischemically damaged rat livers by chromatographies on Sephacryl S-300, CM Sepharose, hydroxyapatite, and Superose 12. The LDGIF was isolated with an overall purification of 194-fold and 40% recovery. Although LDGIF was identified as the rat liver arginase by N-terminal sequence analysis, LDGIF exists as a monomer and the purified native arginase has a trimer form. Furthermore, LDGIF has a lower enzyme activity on the hydrolysis of L-arginine and a higher inhibitory effect on proliferation of FSC than the normal rat liver arginase. The catalytic activity of LDGIF is ascribed to the monomeric characteristics of the LDGIF. Therefore, the inhibitory action of LDGIF might not be due to the arginine depletion by the catalytic activity of arginase. In conclusion, the presence of the LDGIF could interpret the clinical course that serious fibrosis is not found in the liver of patients recovering from severe hepatic necrosis due to fulminant hepatitis, suggesting that this LDGIF may provide a novel target for the prevention and treatment of hepatic fibrosis.

Keywords: Fat-storing Cells; Fulminant hepatitis; Liver perfusates; Monomeric arginase

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Introduction

Acute liver failure most commonly results from acute massive liver cell necrosis caused by viral hepatitis, toxic drugs, and chemicals. Acute liver failure is a devastating complication of acute viral hepatitis; encephalopathy, coagulopathy, renal and cardiovascular changes (McIntyre, 1990; Lee, 1993). The clinical feature of acute viral hepatitis varies widely from asymptomatic to fulminant, and the final outcome is complete recovery, death, chronic hepatitis or cirrhosis. A fulminant course is characterized by acute liver failure associated with massive necrosis and a high mortality (Lidofsky, 1995; Bhaduri and Mieli-Vergani, 1996). Importantly, regardless of the cause, neither chronic process nor fibrotic change was observed in the liver of patients recovering from severe hepatic necrosis due to fulminant hepatitis (Redeker, 1975; Nielsen, 1976). In addition, it has been reported that a factor released from the warm ischemically damaged livers of normal and dimethylnitrosamine-treated animals exhibits not only unspecific immunosuppressive potency but also strong inhibitory properties in fibroblast proliferation *in vitro* (Lie *et al.*, 1987). These results suggest that the acutely injured livers have a factor that allows a recovery from chronic hepatitis or cirrhosis. We assumed that a factor would be released into circulation by massive necrosis of hepatocytes. Thus, the factor could inhibit the proliferation of fat-storing cells (FSC; also referred to as the hepatic stellate cells, lipocytes, Ito cells) which are key effective cells in the progression of liver fibrosis (Gressner, 1998; Friedman, 2000).

It is generally known that liver extracts inhibit mitogenic and allogenic antigen stimulation, and the livers possess endogenous immunosuppressive activities such as antigen unresponsiveness and transplantation tolerance (Kamada, 1985; Hahm *et al.*, 1995). The liver-derived immunosuppressive factor inhibiting lectin-induced lymphocyte proliferation and mixed lymphocyte reaction *in vitro* has been identified as arginase (Schmacher *et al.*, 1974; Brusdeilins *et al.*, 1983). In addition, some reports have shown that a potent

growth-inhibitory effect is predominantly mediated by arginase (Holley, 1967; Terayama *et al.*, 1982). The inhibition is dose-related, reversible, and noncytotoxic. The inhibitory effect of arginase in cell cultures has been suggested to be due to depletion of L-arginine in the culture media (Holley, 1967). However, the wide distribution of arginase among many tissues (Glass and Knox, 1973) and the heterogeneity of arginase (Herzfeld and Raper, 1976; Spolarics and Bond, 1988) seem to suggest that arginase may has physiological role other than the function as a key enzyme in the urea cycle. For example, many studies suggested that high serum arginase levels in patients with gastric, colorectal, and breast cancer play a biologically important role against host defense mechanism (Leu and Wang, 1992; Straus *et al.*, 1992; Wu *et al.*, 1994). Furthermore, it has been reported that a balance between arginase and nitric oxide synthase pathway of arginine metabolism plays a key role in regulation of injury, hemodynamics, and mesangial cell proliferation (Cook *et al.*, 1994).

In this present study, we prepared perfusates by *in vitro* perfusion of the warm ischemically damaged liver and used the perfusates as a source of anti-fibrotic factor. We also report the purification and partial characterization of a liver-derived growth inhibitory factor (LDGIF) that inhibits the proliferation of FSC.

Materials and Methods

Preparation and characterization of liver perfusates Male Sprague-Dawley rats (250 to 300 g) were given food and water freely before surgery. Liver perfusion was performed according to the method described by Varin and Huet (1985) with modification. The animals were anesthetized with pentobarbital (50 mg/kg, i.p.). The portal vein and the superior vena cava were cannulated after ligation of the inferior vena cava and hepatic artery. The livers were flushed with 400 ml of Ringers solution (8.6 g of NaCl, 0.3 g of KCl, and 0.33 g of $CaCl_2$ in 1 liter of distilled water) at a flow rate of 20 ml/min. The livers were removed from the animal and kept at 25°C for 6 h to induce warm ischemic damage. The livers were then perfused with 100 ml of Ringer's solution at a flow rate of 20 ml/min by repeated circulation (10 times). The effluates were collected and used for the experiments. To elucidate the susceptibility of the inhibitor to a denaturing condition or protease digestion, the liver perfusates were treated with acetic acid, heating, trypsin or pronase. After each treatment, the liver perfusates were dialyzed against phosphate buffered saline (PBS) using dialysis membrane (molecular weight cut-off 12 kDa, Sigma, St. Louis, USA).

Isolation and culture of FSC FSC were purified from the non-parenchymal cell suspension by a single step density gradient centrifugation with Nycodenz (5-(N-2,3-dihydroxypropylacetamido)-2,4,6-triido-N,N'-bis(2,3-dihydroxypropyl)-sophthalamide; Sigma) (Schafer *et al.*, 1987; Alpini *et al.*, 1994). The FSC were identified by their typical light microscopic appearance and immunofluorescence staining for desmin (Sigma) (Yokoi *et al.*, 1984). The mean purity of freshly isolated cells as analyzed by fluorescence-activated cells sorting (FACS Calibur, Becton Dickinson, San Jose, USA) was 85 ± 5%, cell viability as checked by Trypan blue exclusion was 90 ± 5%, and the yield ranged from 12 to 20×10^6 cells/liver. The cells were seeded with a density of 1×10^5 cells/cm^2 and maintained with Dulbecco's modified Eagle medium (DMEM) containing 4 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), 10% fetal bovine serum (FBS, GIBCO-BRL, Gaithersburg, USA) and cultured in a humidified atmosphere of 5% CO_2-95% air. The first change of the medium was made 16 h after seeding, after which the purity of FSC was greater than 95%. The medium was changed every 24 h.

Proliferation assay of FSC The assay of the inhibitory activity was performed using FSC as target cells. Experimental manipulations were performed with cells at passages 5-12. FSC were seeded in 24-well tissue culture plates in a volume of 1 ml of the culture media containing 10^5 cells/well. After 12 h, the plating medium was changed to culture medium containing perfusate-derived fractions that had been serially diluted. Cells were pulse-labeled the [^{3}H] thymidine (Amersham, Buckinghamshire, UK) for 24 h before harvesting. All cultures were performed in triplicate and the inhibition of proliferation was calculated as follows: Inhibition (%) = (1-cpm of experimental culture/cpm of control culture) × 100. One unit of LDGIF activity in each separation step was defined as the amount of protein that led to 50% inhibition of proliferation of FSC under the conditions described above.

Purification and characterization of LDGIF All purification procedures were carried out at 4°C. About 2000 ml of liver perfusates, prepared as described above, were concentrated 100-fold with ultrafiltration (molecular weight cut-off 10 kDa, Amicon, Beverly, USA). The concentrates were dialyzed against buffer A (20 mM sodium phosphate, pH 7.0, 5 mM EGTA, 0.02% NaN_3). The solutions were loaded onto a 2.6 × 100 cm Sephacryl S-300 (Pharmacia, Uppsala, Sweden) column equilibrated with buffer A containing 100 mM NaCl and then eluted with the same buffer at a flow rate of 20 ml/h. The eluates were assayed for inhibitory activity on proliferation of FSC as described above. The active fractions were pooled, dialyzed against buffer B (20 mM sodium phosphate, pH 6.0), and loaded onto a CM Sepharose column (2.6 × 10 cm) equilibrated with buffer B. The column was washed with buffer B, and developed with a linear NaCl gradient from 0.1 to 1 M. Fractions were collected and aliquots were assayed for the inhibitory activity. The active fractions were applied to a hydroxyapatite column (1.0 × 5 cm, Bio-Rad, Hercules, USA) equilibrated with buffer B. The charged column was eluted with a stepwise gradient from 0 to 0.5 M sodium phosphate buffer pH 6.0 by 50 mM interval at a flow rate of 0.5 ml/min. The active fractions of eluates were pooled, concentrated with Centriprep 10 (molecular weight cut-off 10 kDa, Amicon), filtered, and loaded for fast protein liquid chromatography onto a Superose 12 column (1.0 × 30 cm, Pharmacia) at a flow rate of 0.25 ml/min. The active fractions, designated as LDGIF, were used for further characterization of its inhibitory principle as described below. On the other hand, arginase was purified from

rat livers according to the methods of Schimke (1964) and Tarrab *et al.* (1974).

Arginase activity assay and arginine supplement test The arginase assay was performed with the method of Schimke (1964). One unit of arginase activity is equivalent to 1 mmol of urea produced min^{-1} at 37°C (Archibald, 1945). The effect of arginine on the inhibitory activity of LDGIF was performed with an arginine supplement test. FSC (10^5 cells) were treated for 10 min in DMEM (2% FBS) supplemented with LDGIF, arginase, or different arginine concentrations, the residual concentration of arginine in the conditioned medium was measured. After the plate was washed with PBS, cells were incubated in fresh DMEM (10% FBS) with [^{3}H] thymidine for 24 h and assessed for the growth of FSC by thymidine incorporation assay. For comparison of the catalytic activity between arginase and LDGIF, 1 μg LDGIF or arginase was added in incubation mixtures containing 100 mM L-arginine, 50 mM Tris·HCl buffer pH 8.8 with 0.1 mM $MnCl_2$ or without $MnCl_2$. Arginase activity was determined as described above.

Cell lines and culture Growth inhibitory effects of arginase and LDGIF were assayed against various mammalian cells. The cell lines used were as follows: 3T6 (mouse embryo fibroblast), HepG2 (hepatocellular carcinoma), CHO (hamster ovary fibroblast), HEK (human embryonic kidney fibroblast), WISH (human amnion epithelial cell), MDBK (bovine kidney epithelial cell), VERO (monkey kidney epithelial cell), LU (human lung fibroblast), KIMA (human kidney fibroblast), and UMR (rat osteogenic sarcoma). The cells were cultured in suitable media, which are used conventionally in many laboratories.

General analytical procedures Electrophoresis was carried out on a pre-made 4-20% gradient Tris-glycine gel (Novex, San Diego, USA) by the procedure of Laemmli (1970). Isoelectric focusing (IEF) was done using pre-made pH 3-10 gel according to the manufacturers instructions (Novex). Protein contents were determined with a dye-binding assay using Coomassie brilliant blue G-250 (Bradford, 1976). For immunoblot analysis, LDGIF and arginase were separated on 4-20% gel, transferred to a nitrocellulose membrane, and incubated with using anti-bovine liver arginase antibody (Rockland, Gilbertsville, USA). Denatured M_r of LDGIF was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and native M_r was estimated by high performance liquid chromatography (HPLC) system (Phamacia-LKB model). In HPLC, the purified form of LDGIF was applied to TSK G3000SW column (7.5 × 600 mm, Tosoh, Tokyo, Japan) equilibrated with 50 mM sodium phosphate buffer pH 7.5 containing 150 mM NaCl and monitored at a wavelength of 280 nm. M_r determination by gel filtration was carried out by measuring the elution volumes of several standard substances. For sequence analysis, the purified LDGIF was electrophoresed on SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad) (Matsudaira, 1987). The corresponding protein band is excised with a scalpel and the N-terminal amino acid sequence was determined by an automatic 447A Protein Sequencer/120A Analyzer (Applied Biosystems, Foster City, USA). Residual arginine in a culture medium was determined by routine method of amino acid analysis. Briefly, after removal of protein by Sephadex G-25 column, the conditioned medium was dried and derivatized with phenylisothiocyanate (PITC). The PITC-amino acid derivatives were then analyzed on reverse phase C18 column by the HPLC system.

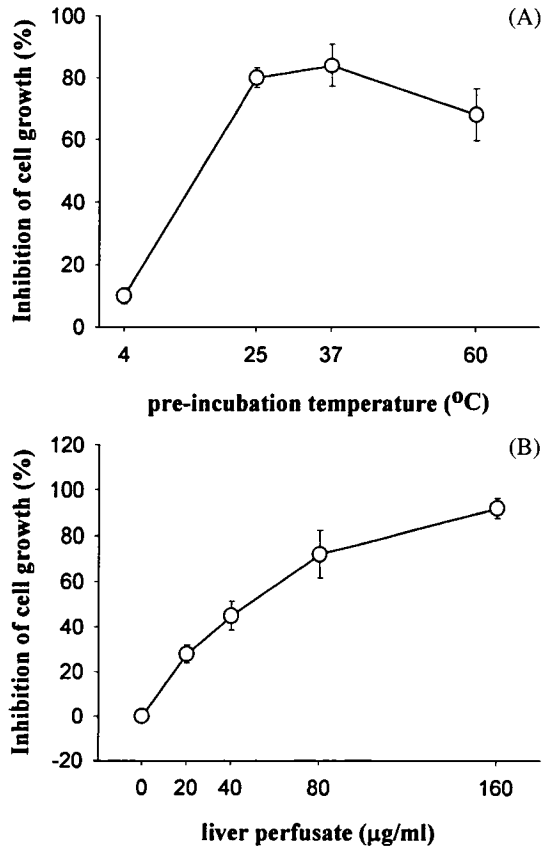


Fig. 1. Growth inhibitory effects of liver perfusates on FSC. (A) Determination of pre-incubation temperature for the production of growth inhibitory factor. After removing blood from the livers, the livers were removed from the rat and kept at the indicated temperature for 6 h to induce ischemical necrosis. The livers were then perfused by repeated circulation and the effluates (70 μg) were used for the proliferation assay of FSC. (B) Dose-dependent effect of liver perfusates on the proliferation of FSC. The liver perfusates were prepared by ischemical necrosis. FSC were seeded in 24 well tissue culture plates with a density of 10^5 cells/well. After 12 h, the indicated amount of liver perfusates was added. The measurement of FSC proliferation was determined with thymidine incorporation for 24 h. Results are expressed as mean ± SE of three separate experiments.

Results

Preparation and characterization of liver perfusates In the present investigation, liver perfusates were prepared by *in vitro* perfusion of the warm ischemically damaged livers and examined for the inhibitory activity on the proliferation of

Table 1. Growth-inhibitory activity of liver perfusates

Treatment[a]		Growth inhibition[b] (% of control)
Perfusates (control)		100
Incubated with	trypsin	6.3 ± 0.9
	pronase	3.8 ± 0.6
	acetic acid	2.2 ± 1.0
Heated at	100°C for 5 min	2.6 ± 1.2
	60°C for 30 min	82.3 ± 2.5
	37°C for 60 min	95.0 ± 2.7

[a]The amount of liver perfusates under denaturing conditions was approximately 200 μg. 0.1 M acetic acid was used for acidification at pH 3.5 for 10 h and heat denaturation was performed at 37, 60, and 100°C. For protease digestion, the liver perfusates were treated in 8 M urea containing 5 mM 1,4-dithiothreitol at 25°C for 4 h followed by 4-fold dilution with 50 mM Tris-HCl pH 8.0 containing 10 mM iodoacetamide. 2 μg of trypsin or pronase was added and digestion was performed at 37°C for 10 h. After each treatment, the liver perfusates were dialyzed against PBS. FSC were adjusted to 10^5/ml in culture medium and 50 μg of test samples was added to each well.

[b]The inhibitory activity was determined by thymidine incorporation. Results are expressed as mean ± SE of three separate experiments.

FSC. After isolated livers were kept at various temperatures for 6 h, the livers were then perfused by repeated circulation, and the liver perfusates were examined. As depicted in Fig. 1A, the growth inhibitory activity gradually increased up to the incubation temperature of 37°C. However, the inhibitory activity declined above 37°C. The liver perfusates inhibited the cell growth of FSC in a dose-dependent (Fig. 1B). To clarify further the chemical nature of the inhibitory factor, we tested the effects of various treatments on the inhibitory activity of the liver perfusates. As shown in Table 1, the factor proved to be protease-, heat- and pH-sensitive. On the basis of these criteria, we assumed that the liver-derived growth inhibitory factor (LDGIF) is a protein.

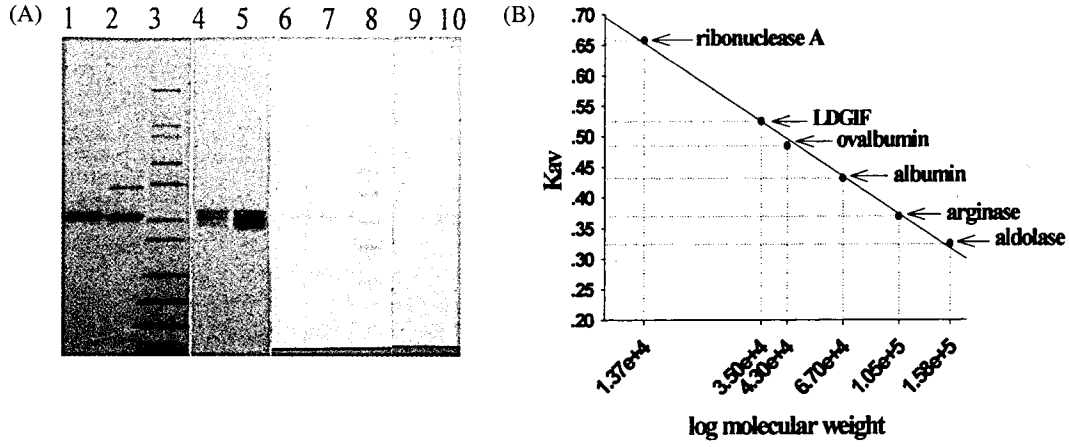
Purification of LDGIF The purification of the growth inhibitory factor was attempted using 2000 ml of perfusates prepared from 20 normal rats as the starting material. The materials were concentrated to 20 ml by ultrafiltration using a YM 10 membrane (at this point, the filtrates do not have any inhibitory effect on the cell growth of FSC). The concentrates were dialyzed and subjected to a Sephacryl S-300 column chromatography. The fractions of growth inhibitory activity were collected (elution volume = 340 ± 40 ml), dialyzed, and then applied to CM Sepharose column. The growth inhibiting activity was eluted at NaCl concentrations from 0.15 to 0.3 M. The active fractions eluted with the linear NaCl gradient were combined and applied to a hydroxylapatite column. The growth inhibitory activity was absorbed on the column and eluted with stepwise sodium phosphate gradient at intervals of 50 mM. Eluates were collected as 0, 50, 100, 150, 200, 250, 500 mM sodium phosphate fractions, and each fraction was tested for its ability to inhibit FSC proliferation. 150, 200, 250, and 500 mM sodium phosphate fractions all contained inhibitory activity (50% inhibitory activity = 19.0, 4.8, 0.5, and 5.6 μg/ml respectively) but 250 mM fractions have the highest inhibitory activity. This chromatography step effectively separated the growth inhibitor from most of the contaminating proteins, but the purification yield was low in this step (a decrease in yield of 37.6% against the previous step). The active fractions were pooled, concentrated to 0.2 ml with a Centricon 10, and subjected to FPLC on a Superose 12 column. The growth inhibitory activity was recovered coincidentally with a protein peak. The purification of LDGIF is summarized in Table 2. About 1 mg of LDGIF was isolated with overall purification of 194.4-fold and 39.7% recovery.

Table 2. Purification of LDGIF from 2000 ml of liver perfusates

Step	Total protein (mg)	Total activity (units)	Specific Activity[a] (units/mg)	Recovery (%)
Liver perfusates	520	12000	23.1	(100)
Sephacryl S-300	204	10920	53.5	91.0
CM Sepharose	32.1	9370	291.9	78.1
Hydroxyapatite	2.88	5840	2027.8	48.7
Superose 12	1.06	4760	4490.6	39.7

[a]One unit of inhibitory activity is defined as the amount of protein necessary to inhibit 50% of FSC growth at 10^5 cells per well after 24 h incubation in DMEM containing 10% FBS.

Characteristics of LDGIF The cell growth of FSC was inhibited in a dose-dependent manner by LDGIF. The inhibition by around 50% was obtained at 0.22 μg protein of the inhibitor per ml of control medium after incubation for 24 h. At the concentration of 0.4 μg per ml, more than 80 % of the cell growth was inhibited in comparison with the control. When analyzed by SDS-PAGE under reducing and non-reducing condition, the purified inhibitor showed single proteins' band with M_r of approximately 35 kDa under reducing condition, whereas the LDGIF showed three bands with M_r of approximately 33, 35, and 38 kDa under non-reducing condition (Fig. 2A). Because the four protein bands have the same N-terminal sequences in sequence determination, these bands may represent variably degraded forms at C-terminals. The N-terminal amino acid sequence data of the LDGIF revealed high homology with that of rat liver arginase (Fig. 2C). In order to test whether the LDGIF has the same general physicochemical properties as arginase, we isolated arginase from rat liver extracts and compared its characteristics to LDGIF. As shown in Fig. 2A, M_r of arginase is 35 kDa and 33/35/38 kDa under reducing and non-reducing



(C) Rat arginase MSSKPKPIEIIGAPFSK

LDGIF MS-KPKPIEIIGAP

Fig. 2. Physicochemical properties of LDGIF and arginase. (A) SDS-PAGE (lane 1-5) and Western blot (lane 6-10) of LDGIF and rat liver arginase under reducing (R) and non-reducing (N) conditions. Lane 1, LDGIF (R); lane 2, rat liver arginase (R); lane 3, protein standard markers (from top, 200, 116.3, 97.4, 66.3, 55.4, 34.5, 31, 21.5, 14.4, and 6 kDa); lane 4, LDGIF (N), lane 5; rat liver arginase (N), lane 6; LDGIF (R); lane 7, rat liver arginase (R); lane 8, protein standard markers (from top, 250, 98, 64, 50, 34, 30, and 16 kDa); lane 9, LDGIF (N), lane 10; rat liver arginase (N). (B) Molecular weight determination of LDGIF and arginase by HPLC system. It was carried out by measuring the elution volumes of several standard substances, calculating their corresponding K_{av} values ($K_{av} = V_e - V_o / V_t - V_o$, V_e: elution volume for the protein, V_o: column void volume, V_t: total bed volume), and plotting their K_{av} values versus the logarithm of their M_r. (C) Comparison of the NH_2-terminal sequence of LDGIF and arginase. The N-terminal sequence was determined by an automatic 447A Protein Sequencer/120A Analyzer.

condition of SDS-PAGE respectively, which is same as the cases of LDGIF. Furthermore, Western blot analysis with a specific antibody against anti-bovine arginase demonstrated that reactivity of LDGIF is similar to that of arginase (Fig. 2A). However, the apparent M_r of LDGIF on TSK G3000SW column was 35 kDa, which is much smaller than 105 kDa of rat liver arginase (Fig. 2B). As shown in Table 3, the LDGIF had a higher FSC inhibitory activity (0.22 μg/ml) than normal rat liver arginase (1 μg/ml). In addition, analysis for the effect of $MnCl_2$ on the catalytic activity of LDGIF indicates that the emzyme activity of LDGIF on the hydrolysis of L-arginine has a much lower $MnCl_2$ dependency (1100 U/mg) than rat liver arginase (5000 U/mg). These results suggest that LDGIF is a monomeric form of arginase having an altered activity.

Effect of arginine on the growth inhibitory activity of LDGIF If the inhibitory effect of LDGIF on FSC growth is similar to that of native arginase, the inhibition may be due to arginine depletion in a conditioned medium. To clarify the effect of arginine on the proliferation of FSC, we tested relationships between the degree of FSC growth and the concentration of arginine in cultured broth after treatment with LDGIF or arginase for 10 min. Regardless of the presence of arginine in conditioned medium, the growth of FSC was inhibited by LDGIF (Fig. 3). The concentration of residual arginine in a medium supplemented with exogenous LDGIF were as follows; 0.40 mM in a medium without arginine supplement, 2.21 mM in a medium with 2.5 mM arginine, and 4.72 mM in a medium with 5 mM arginine. Therefore, the inhibition of growth of FSC by LDGIF is not due to arginine depletion in a cultured medium by the catalytic activity of arginase.

Table 3. Comparison of biochemical properties of LDGIF and arginase

Properties	Arginase[a]	LDGIF[a]
M_r (SDS-PAGE, reducing)	35 kDa	35 kDa
M_r (SDS-PAGE, non-reducing)	33/35/38 kDa	33/35/38 kDa
M_r (gel-filtration)	105 kDa	35 kDa
pI	8.8-9.5	8.8-9.5
Inhibitory activity[b]		
(ED_{50}; μg/ml)	1	0.22
Arginase activity[c]		
with $MnCl_2$ (U/mg)	5,000	1,100
without $MnCl_2$ (U/mg)	250	240

[a]Arginase and LDGIF were purified to the homogenous form from normal liver extracts and ischemically damaged liver perfusates, respectively.

[b]ED_{50} is the concentration required for 50% growth inhibition of FSC. 10^5 cells per well were incubated with LDGIF for 24 h in DMEM containing 10% FBS.

[c]One unit of arginase activity is equivalent to 1 μmole of urea produced min^{-1} at 37°C.

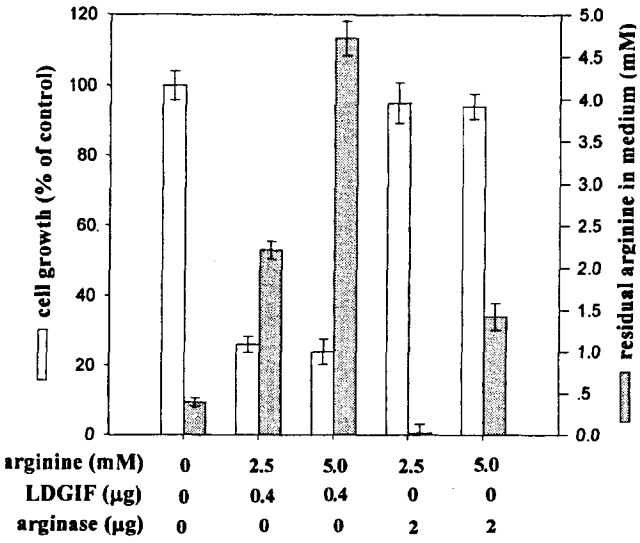


Fig. 3. Characterization of inhibitory activity of LDGIF in the presence of arginine. FSC (10^5 cells) were cultured in DMEM containing 10% FBS for 12 h, washed with PBS, and treated with DMEM containing 2% FBS supplemented with 0.4 μg LDGIF, 2 μg arginase, or different arginine concentrations for 10 min. After the transient treatment, the cultured media were harvested and the residual concentrations of arginine in conditioned media were measured by amino acid analysis. After the plate was washed with PBS, cells were cultivated in fresh DMEM for 24 h and assessed for the growth of FSC by thymidine incorporation assay. Results are expressed as mean ± SE of three separate experiments.

Table 4. Growth inhibition of various mammalians cells in the presence of LDGIF and arginase.

Cell Type	Growth inhibition (%)[a]	
	LDGIF (0.4 μg)	arginase (2 μg)
FSC (fat-storing cell)	80.3 ± 3.2	79.7 ± 4.1
3T6 (mouse embryo fibroblast)	84.7 ± 3.8	81.6 ± 6.1
CHO (hamster ovary fibroblast)	77.3 ± 4.1	75.8 ± 3.5
Lu (human lung fibroblast)	73.7 ± 3.2	70.3 ± 6.2
KIMA (human kidney fibroblast)	68.8 ± 3.5	75.5 ± 2.0
WISH (human amnion epithelial)	36.4 ± 5.8	72.3 ± 4.3
MDBK (bovine kidney epithelial)	42.5 ± 6.9	77.7 ± 3.5
VERO (monkey kidney epithelial)	38.5 ± 1.6	76.1 ± 2.4
HepG2 (hepatocellular carcinoma)	54.2 ± 2.9	74.9 ± 1.5
HMR (rat osteogenic sarcoma)	42.7 ± 6.4	71.3 ± 2.8

[a]Inhibition (%) = (1-cpm of experimental culture/cpm of control culture)×100. Results are expressed as mean ± SE of three separate experiments.

Effects of LDGIF and arginase on the growth of various cell lines We examined the effects of LDGIF and arginase on the growth of various cell lines. At the concentration of 0.4 μg of LDGIF and 2 μg of arginase, 80% of the FSCs growth was inhibited in comparison with the control. As shown in Table 4, LDGIF effectively inhibited the growth of fibroblasts (3T6, CHO, LU, KIMA) more than epithelial cells (WISH, MDBK, VERO) and tumor cells (HepG2, HMR), whereas the arginase had a similar sensitivity against various cell lines tested with 70-80% inhibitions at 2 μg of arginase.

Discussion

Our approaches to intervention of chronic liver disease were based on the facts that hepatic fibrosis is not observed in patients who recover from fulminant hepatitis, and *in vivo* damage of parenchymal cells by acute hepatitis is similar to that of liver cells by ischemical perfusion *in vitro* (Lie *et al.*, 1987). Through the inhibition assay on the proliferation of FSC, we isolated a growth inhibitory factor designated as LDGIF. LDGIF has been identified as a monomeric form of arginase and as having a higher inhibitory effect and lower arginase activity than the native arginase. The LDGIF released from the liver by warm ischemical perfusion probably exists as a monomer, which clearly distinguishes it from trimer form of native arginase (Berueter *et al.*, 1978; Kanyo *et al.*, 1992). It also discriminates arginase from rat liver perfusates (Chisari *et al.*, 1985) or the liver-type arginase emerged in serum after reperfusion of the liver graft (Ikemoto *et al.*, 1998). This discrepancy may be due to the degree of hepatocyte damage that standing of liver at 25°C for 6 h, which induces massive necrosis in our perfusion system. Thus, the LDGIF isolated from damaged livers by ischemical necrosis exists a monomeric arginase, whereas the native form of arginase isolated from normal livers is a trimer. Generally, arginase specifically requires a spin-coupled Mn^{++}-cluster for catalytic activity *in vitro* and *in vivo* (Kanyo *et al.*, 1996), and dissociated arginase (monomer) showed no enzyme activity (Hirsch-Kolb and Greenberg, 1968). Furthermore, dialysis of arginase with chelating agents caused the trimetric enzyme to dissociate fully into monomer (Green *et al.*, 1990). Although the LDGIF releasing condition is not similar to the dialysis condition, the low arginase activity of LDGIF may be due to the monomeric nature of LDGIF.

Whether the LDGIF has higher cell growth inhibitory activity than native arginase remains to be determined. Since LDGIF is a monomeric arginase, the inhibitory action of LDGIF is not due to the depletion of arginine by the catalytic action of arginase. The fact that the addition of arginine to the culture medium did not reduce the inhibitory action of the LDGIF suggests other cell growth inhibition mechanisms. On

the other hand, LDGIF exhibits more growth inhibitory effects on fibroblasts than epithelial cells. These results suggest the possibility that LDGIF may be used as a therapeutic agent for cirrhosis. At present, we are investigating the action mode of LDGIF on cell growth and preparing the large-scale production of LDGIF for efficacy testing in CCl_4-induced cirrhosis.

In conclusion, we isolated LDGIF from the rat livers by *in vitro* perfusion of ischemically damaged livers. The LDGIF is a monomeric form of arginase with low catalytic activity and high inhibitory activity against FSC growth in comparison with the native arginase. Discovery of the LDGIF could explain the clinical observation that serious fibrosis is not found in the liver of patients recovering from severe hepatic necrosis due to fulminant hepatitis.

Acknowledgments We thank Tae-Yoen Lim (Department of Biochemistry, College of Science and Bioproducts Research Center, Yonsei University) for assistance in the preparation of this manuscript. The study was supported by grants from the Mogam Biotechnology Research Foundation and performed by the KIST-Academica Collaborative Education Program.

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